HAIR FOLLICE AS A SOURCE FOR BIOMARKERS ASSOCIATED WITH LOW-LEVEL VAPOR EXPOSURE TO GF AND VX IN THE RAT AND MINIPIG MODEL

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ABSTRACT

During the aftermath of the 1995 subway terrorist attack in Tokyo, the thousands of individuals who sought treatment completely overwhelmed resources at local emergency rooms. Of these patients, those who thought they had been exposed to nerve agent outnumbered those who were truly exposed by a ratio of five to one. The most common test for OP compound exposure is measurement of blood acetylcholinesterase. Unfortunately, this blood test is neither rapid, nor can it provide a uniformly reliable assessment of low-level exposure to organophosphate compound in this scenario. Thus, a rapid, reliable, and non-invasive test would be invaluable for the effective management of medical resources in the event of a similar civilian terrorist incident in the U.S. In order to address this need, we have begun a series of experiments designed to identify protein biomarkers in follicles (hair and whisker) of animals exposed to low levels of OP nerve agents.

1. INTRODUCTION

At 8:05 am on March 20, 1995, members of the fanatic cult AUM Shinrikyo released sarin nerve gas on three different Tokyo subway lines. Within 15 minutes of the agent release, injuries were reported in 15 of Tokyo's underground railroad stations and seven people were reported dead at the site. The entire Tokyo metropolis went into a panic and area hospitals began to flood with people, both exposed and people fearing they had been exposed[1]. Since the terrorist attacks of September 11, 2001, officials have become increasingly worried about the release of chemical or biological agents in populous civilian areas. Even if major causalities weren't sustained, an attack would overwhelm first responders and hospitals as people, fearing they have been exposed, rush to seek medical attention.

As was the case in Japan, an actual, or even perceived, terror attack in the U.S. would overwhelm first responders and the medical community. In the Tokyo sarin attack, an estimated 5,000-6,000 people were exposed to sarin. Over half of these people (3,227) flocked to all 41 of Tokyo's hospitals. Less than

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15% of those people who went to the hospital needed to be admitted and only 0.5% (17 people) required intensive care[2].

St. Luke's International Hospital in Tokyo reported that 641 patients came to the emergency room for possible sarin exposure. Approximately 83% of these patients were treated as outpatients. These outpatients were displaying the eye problems that are associated with sarin exposure. However, when given intravenous doses of atropine sulfate, they had no reaction to the antidote. Several patients even complained of heart palpitations after the atropine sulfate was administered. The lack of response of the outpatients to the atropine sulfate, the antidote commonly given for sarin exposure, indicates that these individuals might not have been exposed to sarin even though they were suffering the common eye problems[3].

One of the major problems that occurred with treating patients was due to the fact that doctors weren't sure what the patients had been exposed to, although all the systems indicated some kind of nerve agent. It was not confirmed that sarin was released until after blood tests confirmed that acetylcholinesterase levels were decreased and sarin hydrolysis products were present. Due to the lack of knowledge about what was initially released, first responders, police, doctors and hospital staff did not take precautionary measures and they too began to suffer symptoms of exposure[4].

From the tragic events that occurred in Japan, there are lessons to be learned from the situation and its aftermath. One of the most important lessons is that a rapid test needs to be developed that can very quickly determine whether individuals have actually been exposed to nerve agent or are simply experiencing post traumatic anxiety induced symptoms. Currently, measurement of acetylcholinesterase levels in the blood is the fastest way to determine exposure. Although this test is faster than looking for hydrolysis products in the urine or blood, there are still several drawbacks to the test due to the inherent nature of acetylcholinesterase. First, there is 10-18% interindividual variation and a 3-7% intraindividual variation for acetylcholinesterase levels[5]. Due to such variations, without a known baseline, it is hard to definitively conclude if a person has been exposed to a nerve agent if they show inhibition levels of less than 20%. Secondly, due to the *de novo* synthesis of new enzyme, this method cannot be used for retrospective determination of exposure[6]. Thus, there is no technique currently available to rapidly determine exposure to chemical warfare agents.

In this work, we will identify protein biomarkers indicative of chemical warfare agent exposure and will use layered expression P-FILM membranes to monitor the exposure to chemical warfare agents using the hair follicle.

2. METHODS

2.1. OP Nerve Agents

Sarin (GB) of 98.16 +/- 0.36% purity and VX of 93.6 +/- 0.5% purity were obtained from the US Army Edgewood Chemical Biological Center. All purities were confirmed by NMR analysis.

2.2. Exposures to and Monitoring of VX Exposure in the Rat

Male and female Sprague-Dawley rats, seven to eight weeks old, (Charles River Laboratories, Wilmington, MA) were confined in individual stainless steel compartmentalized cages (20" x 14" x 4") in a 750 L dynamic airflow inhalation chamber for whole body VX inhalation. The female rats were exposed to 0.000374, 0.00067, 0.00105 or 0.00183 mg/m^3 of vapor for 240 minutes. The male rats were exposed to 0.00061, 0.00067, 0.00105 or 0.00183 mg/m^3 of vapor for 240 minutes. All the animals were monitored during and after the exposure for the clinical signs of lethality and sub-lethality (miosis, tremors, salivation, lacrimation, labored breathing, convulsions). Miosis was the only observed clinical sign. Pupil size was accessed on both pre- and post-exposed animals using an infrared digital camera under low light conditions (less than 10 foot candles). Both acetycholineseterase and butylcholinesterase blood levels were monitored (modified Ellman method) from pre- and post-exposed animals. Blood was obtained from the tail vein. One week after exposure, the animals were killed. Whiskers were pulled manually, with care to remove intact follicles, immediately snap frozen in liquid nitrogen and stored at –135^{o}C until analysis.

2.3. Exposures to and Monitoring of Sarin (GB) Exposure in the Minipig

Male and female Gottingen Minipigs, five to six months old, (Marshall Farms, PA) were confined in slings in a 1000 L dynamic airflow inhalation chamber for whole body sarin inhalation for 10, 60 or 180 minutes. The concentration of sarin was varied per animal, depending on the previous experiments determining the ECT_{50} for miosis. Signs of miosis were continuously monitored for each animal. Pupil size was accessed on both pre- and post-exposed animals using an infrared digital camera under low light conditions (less than 10 foot candles). Both acetycholineseterase and butylcholinesterase blood levels were monitored (modified Ellman method) from pre- and post-exposed animals. Blood was taken from a surgically implanted neck catheter. Hair samples from the area between the shoulder blades were taken from the pigs during surgery and immediately following death. The hair was immediately placed in acetone and then frozen at –80^{o}C for further use.

2.4. Transfer of Hair Follicle Proteins onto P-Film

Two methods were used to transfer the proteins from the hair follicle onto the P-film membranes. Both are described below.

2.4a. *Electrotransfer Method*. Hair samples were fixed for 15 minutes in Acetone and then incubated in PBS for 15 minutes. Filter paper (BioRad, Hercules, CA), 0.45 μm Nitrocellulose (Schleicher and Schuell, Keene, NH) and 5-stack P-Film membranes (20/20 Gene Systems, Rockville, MD) were soaked in Electrotransfer buffer prior to use. PCPVPF membranes (20/20 Gene Systems, Rockville, MD) were used dry. Electrotransfer was performed in a BioRad Mini Trans Blot cell at a constant 1mAmp for 2 hours at 4^{o}C. Following the transfer, the nitrocellulose trap was stained for protein using Fast Stain (Chemicon, Temecula, CA). Manufacturer's instructions were followed. The P-film membranes were dried and saved for further use.

2.4b. *Heat Transfer Method*. Hair samples were fixed for 15 minutes in Acetone and then incubated in PBS for 15 minutes. Hair follicle proteins were transferred from glass slides into 5-stack P-film membranes (20/20 Gene Systems, Rockville, MD) and 0.45 μm nitrocellulose (Schleicher and Schuell, Keene, NH) in Heat Transfer Buffer (20/20 Gene Systems, proprietary) using an 80^{o}C heating surface, light pressure and capillary action for two hours. Following the transfer, the nitrocellulose trap was stained for total protein using Fast Stain (Chemicon, Temecula, CA). Manufacturer's instructions were followed for the Fast Stain. The P-film membranes were dried and saved for further use.

2.5. Total Protein Detection of Membranes

Dried membranes were initially wetted in PBS. The membrane was then washed 3 times, 5 minutes per wash, in PBS. EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL) was dissolved in PBS to a concentration of 4.4 mg/ml. Manufacturer's instructions were followed with slight modifications. After quenching, the membrane was washed 3 times, 5 minutes per wash, with PBS. Cy3 labeled streptavidin (Amersham Biosciences, Piscataway, NJ) was diluted to 1 mg/ml with PBS. The membrane was then incubated with a 1:400 dilution of Cy3 labeled streptavidin for 1 hour at room temperature in darkness. Following incubation, the streptavidin was removed and the membrane was washed 3 times, 5 minutes per wash, with PBS and 5 times, 2 minutes per wash in ddiH_20. The P-film membrane was then scanned in an Affymetrix 428 scanner (Affymetrix, Santa Clara, CA). Images were analyzed on Affymetrix Jaguar 2.0 software (Affymetrix, Santa Clara, CA).

2.6. Specific Protein Detection

2.6a. Antibodies. The following mouse monoclonal antibodies were used in this work: anti-Phospho-VEGF Receptor 2 (Cell Signaling, Beverly, MA), anti-Glutathion Transferase (Chemicon, Temecula, CA), anti-Keratin 10/13 (Neo Markers, Fremont, CA), anti-CD3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Na^{+}/K^{+}ATPase α1 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Na^{+}/K^{+}ATPase β1 (Santa Cruz Biotechnology, Santa Cruz, CA). The following rabbit polyclonal

antibodies were used in this work: anti-Androgen Receptor (AR) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GAPDH (Novus Biologicals, Littleton, CO) and anti-Brain Derived Neutrophic Factor (BDNF) (Santa Cruz Biotechnology, Santa Cruz, CA). The following goat polyclonal antibodies were used in this work: anti-CYP1A1 (Cytochrome P450 1A1) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD20 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ALDH1A2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Glut 1 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-TNFα (Santa Cruz Biotechnology, Santa Cruz, CA). Cy5 labeled goat anti-rabbit IgG and Cy5 labeled goat anti-mouse IgG were purchased from Amersham Biosciences, Pisataway, NJ. Cy5 labeled rabbit anti-goat IgG was purchased from Chemicon, Temecula, CA. Other materials used are listed below.

2.6b. Antibody Blotting. Dried P-film membranes were wetted in TTBS (0.5% Tween, Tris pH 8.0) and then blocked overnight at 4°C in TTBS containing 1% BSA. Following blocking, the P-film membranes were incubated with the primary antibody (typically 1:50 to 1:100) diluted in TTBS containing 0.5% BSA and 0.01% Sodium Azide. The P-film membrane was incubated in primary antibodies for 1 hour at room temperature. The P-film membranes were washed 3 times, 5 minutes per wash, with TTBS at room temperature. Dye-conjugated secondary antibodies (typically 1:200) were diluted in TTBS containing 0.5% BSA and 0.01% Sodium Azide and protected from the light. Secondary antibodies were incubated with the P-film for 30 minutes at room temperature in darkness. After incubation, the secondary antibodies were removed and the P-film membranes were washed 3 times, 5 minutes per wash, in TTBS. The membranes were then washed 5 times, 2 minutes per wash, in ddiH_20. The membranes were scanned and analyzed as described above.

2.6c. SDS-PAGE and Western Blotting of Hair Protein. Protein was extracted from both the shaft and bulb regions from groups of 10 hairs from a single donor animal. The hairs were cut to separate the bulb and shaft regions. Separated bulb and shaft regions were then incubated in PBS containing 1% SDS for 7 minutes at 100°C. The samples were then placed on ice and the protein concentrations were determined using the DC Protein Assay kit (BioRad, Hercules, CA). Extracts were separated by SDS-PAGE using a 15% Tris-HCl Ready Gel containing a 4% stacking gel (BioRad, Hercules, CA). Equal amounts of the extracted hair protein were heated in Sample Buffer (62.5 mM Tris-HCl, pH 6.8 containing 2% SDS, 20% Glycerol and 0.01% Bromophenol Blue) at 100°C for 3 minutes prior to loading. Kaleidoscope markers (BioRad, Hercules, CA) were used as size standards.

Following electrophoresis, the gel was incubated for 15 minutes in Transfer Buffer (25 mM K_2HPO_4, 25 mM KH_2PO_4 and 0.25 mM EDTA). The proteins were then transferred onto 0.45 μm nitrocellulose membrane (Schleicher and Schuell, Keene, NH) in a BioRad Mini Transfer Cell.

After transferring, the nitrocellulose membranes were blocked overnight at 4°C in TBS containing 1% BSA. Primary antibodies were diluted to a concentration of 1:50 to 1:100 in TBS containing 0.5% BSA and 0.01% Sodium Azide. The membranes were incubated with the primary antibody for 1 hour at room temperature. The membranes were washed 3 times, 5 minutes per wash, with TTBS. Dye-conjugated secondary antibodies were diluted in TTBS containing 0.5% BSA and 0.01% Sodium Azide to a concentration of 1:200. The membranes were then incubated in secondary antibody for 30 minutes in darkness. Following removal from secondary antibody, the membranes were washed 3 times, 5 minutes per wash, in TTBS and 5 times, 2 minutes per wash, in ddiH_2O. The membranes were scanned and analyzed as described above.

3. DISCUSSION

Hair analysis has been used for many years to test for chronic exposure to chemicals, most notably drugs of abuse. Over this past year, scientists from the University of Crete have demonstrated that hair analysis can be used to determine chronic exposure to the organophosphate pesticide diazinon. The scientists were testing for the presence diazinon in the hair, not the byproducts of hydrolysis that most hair tests detect. New Zealand white rabbits were given varying concentrations of diazinon in their drinking water for 4 months. Hair was removed from the backs of the animals, extracted and analyzed by HPLC. Serum cholinesterase measurements were also taken. It was found that the more diazinon found

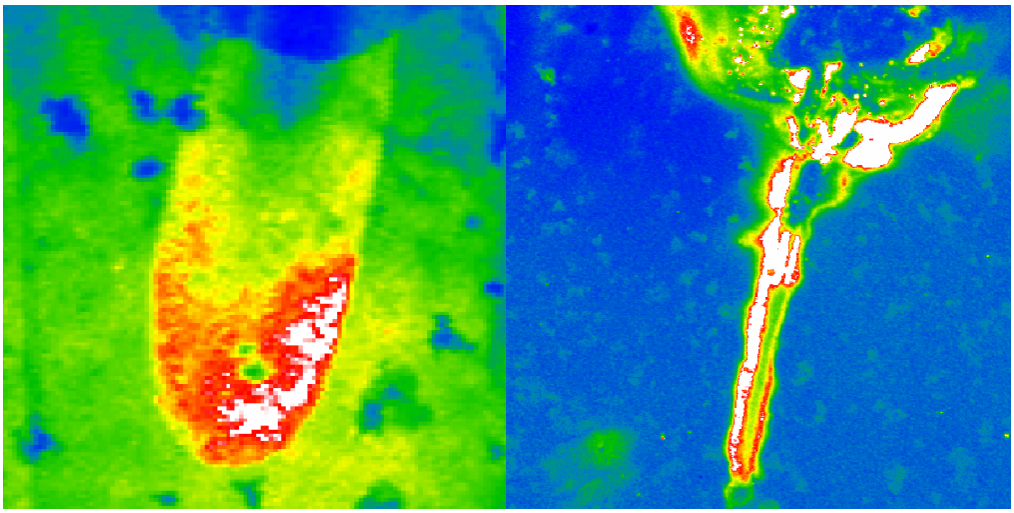

Figure 1: Left Image: Electrotransferred rat whisker follicle probed with anti-Na^+/K^+-ATPase α1. Right Image: Electrotransferred rat whisker follicle probed with anti-Na^+/K^+-ATPase β1.

in the hair, the more decreased the serum cholinesterase levels were[7]. OP pesticides are very similar in structure and mechanism to OP chemical warfare agents like VX, sarin, soman and tabun. The only main difference is that the chemical warfare agents are much more potent than their OP pesticide cousins. Due to the similarities however, we would expect sarin and VX to be found in the hair. This means that all the cells in the hair follicle would be exposed to the agent and should undergo toxic response to the agent.

Due to the evidence in the literature of their involvement in pesticide toxic response, described below are the different categories of biomarkers that we hypothesize to be altered in response to OP chemical warfare agent exposure due to their reactions when exposed to varying pesticides and chemical warfare agents.

3.1. Membrane Bound Targets

The sodium-potassium ATPase is a membrane-bound integral protein found in all tissues involved in the transport of sodium and potassium ions across the plasma membrane. The energy needed to pump out three sodium ions and bring in two potassium ions is provided by the hydrolysis of ATP[8]. The sodium-potassium ATPase consists of multiple subunits, including three isoforms of the α and β subunits[9]. The sodium-potassium ATPase haloenzyme has a molar ratio of 1:1 for the α and β subunits[10-11]. In a limited number of tissues, there is also γ subunit[12].

The three isoforms of the α (α1, α2 and α3) and β (β1, β2 and β3) subunits are encoded by multiple genes. The isoforms are tissue specific and developmentally regulated[13]. The α subunit contains the ATPase activity[14]. For this series of experiments, antibodies towards the α1 and β1 subunits were chosen due to the fact that these isoforms are expressed in most tissues[15].

Several studies have shown that pesticides alter the activity of the sodium-potassium ATPase. Cascorbi and Foret demonstrated in cell culture that the more lipophilic a xenobiotic is, the greater the decrease in the sodium-potassium ATPase activity[16]. Another study using the pesticide pentachlorophenol (PCP) demonstrated that pesticides could inhibit the active transport of sodium across the plasma membrane of toad skin cells[17]. Additionally, it was shown that the cellular demand for oxygen increases upon pesticide exposure. The increase oxygen demand might be indicative of PCP uncoupling oxidative phosphorylation. We hypothesize that we will observe a decrease in the activity of both α1 and β1chains of the sodium-potassium ATPase. Although both of the experiments described above were performed

with organochlorine pesticides, we expect OP nerve agents to have the similar inhibitory effects. Organochlorine pesticides are highly lipophilic, thus they are absorbed easily by the skin and become concentrated in fatty tissues. Sarin and VX, although not very similar in structure to the organochlorine pesticides, are lipophilic and easily absorbed by the skin, indicating that they might have the same effects on membrane bound transporters.

The glucose transporter (GLUT) is another membrane bound transporter whose activity has been shown to be decreased in the presence of lipophilic xenobiotics. As the chief facilitator of transmembrane glucose diffusion, the GLUT is present in at least eight different subtypes through different tissues of the body. Coscorbi and Foret have demonstrated that the more lipophilic the toxicant (i.e. organochlorine pesticides), the more the GLUT activity is inhibited[18]. Though OP nerve agents (VX and sarin) differ significantly in structure to organochlorine pesticides, they are nonetheless lipophilic toxicants. We hypothesized that a change in activity, and potentially level of GLUT 1 (found in keratinocytes) would be found in the VX and sarin exposed animals in our study.

3.2. Immune Response

From other studies, we learned that the immune system appears to play a role in OP nerve agent response. The skin is one of the primary immune response organs in the mammalian system. The mammalian hair follicle is a representative of the skin immune system. In the growth stage, the hair follicle contains Langerhan's cells, $CD4^+$ and $CD8^+$ T cells, macrophages and mast cells. B cells, natural killer and γδ (CD3) T cells are found more rarely in growing follicles[19]. However, Christoph and colleagues were able to show that keratinocytes, that support growth of follicles, are able to promote the growth of Langerhan's cells and tissue resident γδ (CD3) T cells.

Kassa and colleagues have performed several studies on the effects of sarin on the immune system of rats. First, Kassa determined that a single exposure to low-level sarin has a greater effect on the immune system when compared to multiple exposures. Secondly, it has been demonstrated that the number of CD3 cells in the lungs, which represent T-lymphocytes, decreases. Thirdly, it was shown that the number of CD19 cells in the lungs, which represent B-lymphocytes, slightly increase[20-21]. Another group also verified Kassa's findings that sarin suppresses the T-cell response in rats[22].

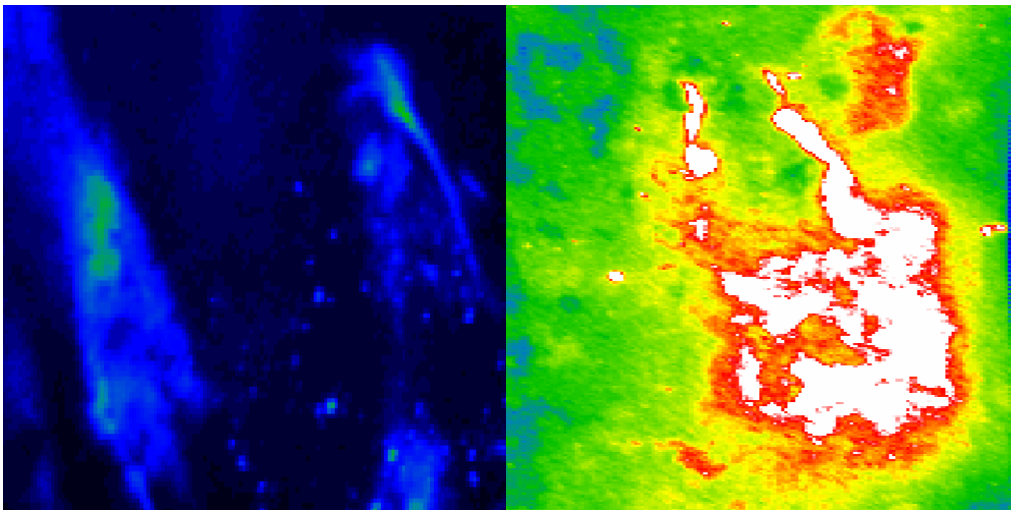

Figure 2: Left Image: Electrotransferred rat whisker follicle probed with anti-CD3. Right Image: Electrotransferred rat whisker follicle probed with anti-Androgen Receptor.

Another immune protein reported to be altered by OP nerve agent exposure is Tumor Necrosis Factor-α (TNF-α). TNF-α is a cytokine released by most types of cells in response to stress. The release of cytokines like TNF-α promotes an anti-inflammatory response by B-lymphocytes. Several studies have been performed in which the levels of TNF-α and other cytokines were measured in the brain tissue of rats after exposure to sarin and soman. It was demonstrated that brain mRNA levels for TNF-α increase after exposure to sarin in both normal and heat-shocked animals[23]. Brain TNF-α mRNA was also shown to be elevated in three regions of the brain in rats exposed to soman[24]. Thus, the increased levels of TNF-α could be part of the same biochemical pathway mechanism that produced the increase in B-cells in response to the sarin observed in the work of Kassa and colleagues work[25].

We are anticipating an increase in the levels of CD19 (representing B-lymphocytes) along with an increase in TNF-α. There should also be a decrease in the amount of CD3 (representing T-cells). Due to the fact that B-lymphocytes and CD3 cells are found more rarely in the hair follicle, there may be practical limitations in detecting changes in these proteins. However, preliminary data has shown the presence of CD3 in the hair follicle. We expect to see the same patterns of increases/decreases as was reported in the literature using sarin due to the fact that VX and sarin are closely related toxicants.

3.3. Hormone Receptors

The hormone receptor class of molecules has been implicated in the response to OP and OC pesticides. Androgens play an important role in the development of the male embryo and spermatogenesis initiation and maintenance[26]. The androgen receptor, along with estrogen and progesterone receptors, regulate gene expression and endocrine function in many cell types[27]. Brakta-Robia and colleagues (2002) and Thornton and colleagues (2003) have demonstrated that the androgen receptor is present in skin cells[28-29]. One study showed that the androgen receptor was present in all the follicular cells except the epidermal cells[30].

Organophosphate pesticides have been shown to have sufficient androgen receptor antagonistic properties[31]. Due to the fact that organophosphorus pesticides are similar in structure and activity to organophosphorus chemical warfare agents, we are anticipating a decrease in the activity and levels of androgen receptor observed in the hair follicle. Organochlorine pesticides also have antagonistic effects on the androgen receptor, although not as great as the effects of the organophosphate pesticides[32].

3.4. Neurotrophins

Brain-derived neutrophic factor (BDNF) is a small polypeptide important for the development, maintenance and regeneration of neurons. In the past few years, there has been increasing interest in the role of neutrophins in the hair follicle. It has been shown that BDNF is found in the hair follicle, but there are conflicting reports on the role it plays in the follicle. Initially, it was reported that BDNF does not appear to influence the morphology or growth of the hair follicle[33]. Later on, the same group reported that BDNF could inhibit the growth of the mature hair follicle and stimulate keratinocyte proliferation[34].

BDNF has been shown to be down regulated in the brain and liver of rats exposed to the pesticide chlorpyrifos[35]. If the BDNF in the hair follicle responds to exposure like the BDNF in the brain and liver did, then we expect to see a down regulation in the hair follicle. Due to the fact that rat whiskers are sensory organs with significant neuronal integration, we expect to see a greater role of BDNF. Although this study performed by exposing the rats to chlorpyrifos, an OP pesticide, we believe that sarin and VX will show similar BDNF inhibition.

3.5. Phase I and Phase II Enzymes

Cytochrome P450 1A1 (Phase I), Glutathion-S-transferase (Phase II) and Aldehyde dehydrogenase (Phase II) are enzymes present in the epidermis that metabolize xenobiotics. The Phase I and Phase II enzymes work together as a complex. This Phase I and Phase II complex is said to be one of the most sensitive cellular responses to xenobiotics[36].

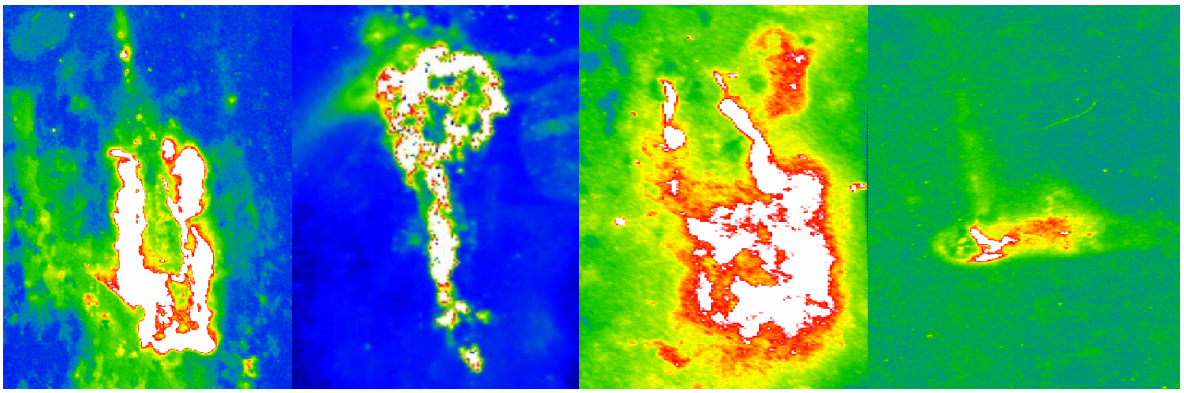

Figure 3: First Image: Electrotransferred rat whisker follicle probed with anti-BDNF. Second Image: Electrotransferred rat whisker follicle probed with anti-Glutathion-S-Transferase. Third Image: Electrotransferred rat whisker follicle probed with anti-Androgen Receptor. Fourth Image: Heat transferred rat whisker follicle probed with anti-Androgen Receptor.

Several studies have been performed that examine the activities of Phase I and Phase II enzymes in response to OP compounds and other classes of pesticides. Three studies have shown that various xenobiotics induce the activity of the Cytochrome P450 1A1, Glutathion-S-transferase and Aldehyde dehydrogenase. Delescluse and colleagues (1998) demonstrated that not only did OP pesticides induce Phase I and Phase II enzyme activities, but pesticides from other classes as well[37]. This indicates that sarin and VX, due to their similarities in structure and activity to OP pesticides, will likely induce Phase I and Phase II activity. Gerlardi and colleagues (2001) and Harris and colleagues (2000) demonstrated that common cytochrome P450 inducers effect the activity of all of the Phase I and Phase II enzymes[38-39].

4. CONCLUSIONS

We have determined that proteins, which in the literature have responded to exposure to pesticides, can be found in the hair follicle. As of today, we have determined that Phospo-VEGF, Keratin 10/13, GAPDH, Glutathion-S-transferase, CD3, BDNF, Androgen Receptor, Sodium-Potassium ATPase α1 and Sodium-Potassium ATPase β1 can be found in the hair follicle. We have tentatively determined that the CYP1A1 is found in the hair follicle. Work is currently being done to determine if CD20, Aldehyde dehydrogenase, Glut 1 and TNF-α are in the hair follicle.

Two transfer methods have been performed in this work. One method involved transferring the proteins by heat and light vacuum action. The second method involved electrotransfer. We have determined thus far that the electrotransfer transfers much larger quantities of protein than the heat transfer and reliably transfers all the hairs to be transferred when compared to the heat transfer. Although greater quantities of proteins are transferred, there is more "bleeding" of the proteins into the surrounding membrane. When the heat and light vacuum are used, less protein is transferred, however there is less bleeding into the surrounding membrane. Currently, we are attempting to optimize the electrotransfer reaction to minimize bleeding and maximize transfer efficiency.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

1. Suzuki, T., Morita, H., Ono, K., Maekawa, K., Nagai, R. and Yazaki, Y. Sarin poisoning in Tokyo subway. *The Lancet* **435**: 980, April 15, 1995.
2. Ohbu, S., Yamashina, A., Takasu, N., Yamaguchi, T., Murai, T., Nakano, K., Matsui, Y., Mikami, R.,Sakurai, K. and Hinohara, S. Sarin poisoning on Tokyo subway. *Southern Medical Journal Online*, June 3, 1997.
3. Ohbu, S., Yamashina, A., Takasu, N., Yamaguchi, T., Murai, T., Nakano, K., Matsui, Y., Mikami, R.,Sakurai, K. and Hinohara, S. Sarin poisoning on Tokyo subway. *Southern Medical Journal Online*, June 3, 1997.
4. Kulling, Per. The terrorist attack with sarin in Tokyo. *Socialstyrlsen* March 20, 1998, pg 68.
5. Maroni, M., Colosio, C., Ferioli, A. and Fait, A. Organophosphorus pesticides. *Toxicology* **143**: 9-37, 2000.
6. Noort, D., Benschop, H.P. and Black, R.M. Biomonitoring of exposure to chemical warfare agents: a review. *Toxicology and Applied Pharmacology* **184**: 116-126, 2002.
7. Tutudaki, M., Tsakalof, A.K. and Tsatsakis, A.M. Hair analysis used to access chronic exposure to the organophosphate diazinon: a model study with rabbits. *Human and Experimental Toxicology* **22**: 159-164, 2003.
8. Lingrel, J.B. and Kuntzweiler, T. Na^+, K^+-ATPase. *Journal of Biological Chemistry* **269(31)**: 19659-19662, 1994.
9. Blanco, G., DeTamaso, A.W., Koster, J., Xie, Z.J. and Mercer, R.W. The α-subunit of the Na,K-ATPase has catalytic activity independent of the β-subunit. *Journal of Biological Chemistry* **269 (38)**: 23420-23425, 1994.
10. Shyjan, A.W. and Levenson, R. Antisera specific for the α1, α2 and α3 and β subunits of the Na^+/K^+-ATPase: Differential expression of α and β subunits in rat tissue membranes. *Biochemistry* **28**: 4531-4535, 1989.
11. Emanuel, J.R., Garetz, S., Stone, L. and Levenson, R. Differential expression of Na+, K+-ATPase α- and β-subunit mRNA's in rat tissue and cell lines. *Proceedings of the National Academy of Sciences USA* **84**: 9030-9034, 1987.
12. Pu, H.X., Cluzeaud, F., Goldshleger, R., Karlish, S.J.D., Farman, N. and Blostein, R. Functional role and immunocytochemical localization of the γ_a and γ_b forms of the Na, K-ATPase γ subunit. *Journal of Biological Chemistry* **276(23)**: 20370-20378, 2001.
13. Mercer, R.W., Biemesderfer, D., Bliss, D.P., Collins, J.H. and Forbush, B. Molecular cloning and immunological characterization of the γ peptide, a small protein associated with Na, K-ATPase. *Journal of Cell Biology* **121(3)**: 579-586, 1993.
14. Blanco, G., DeTamaso, A.W., Koster, J., Xie, Z.J. and Mercer, R.W. The α-subunit of the Na,K-ATPase has catalytic activity independent of the β-subunit. *Journal of Biological Chemistry* **269 (38)**: 23420-23425, 1994.
15. Lingrel, J.B. and Kuntzweiler, T. Na^+, K^+-ATPase. *Journal of Biological Chemistry* **269(31)**: 19659-19662, 1994.
16. Cascorbi, I. and Foret, M. Interaction of xenobiotics on the glucose-transport system and Na^+/K^+-ATPase of human skin fibroblasts. *Exotoxicology and Enviromental Safety* **21**: 38-46, 1991
17. Quevedo, L., Montoya, G., Ferraris, R. and Venegas, W. Inhibition of the sodium transport by pentachlorophenol (PCP) in toad skin (Pleurodema thaul). *Comparative Biochemistry and Physiology Part C* **101(2)**: 365-369, 1992.
18. Cascorbi, I. and Foret, M. Interaction of xenobiotics on the glucose-transport system and Na^+/K^+-ATPase of human skin fibroblasts. *Exotoxicology and Enviromental Safety* **21**: 38-46, 1991
19. Christoph, T., Muller-Rover, S., Audring, H., Tobin, D.J., Hermes, B., Cotsarelis, G., Ruckert, R. and Paus, R. The human hair follicle immune system: cellular composition and immune privilege. *British Journal of Dermatology* 142: 862-873, 2000.

20. Kassa, J., Krocava, Z., Sevelova, I., Sheshko, V., Kassalova, I. and Neubauerova, V. Low-level sarin-induced alteration of immune system reaction in inbred BALB/c mice. *Toxicology* **187**: 195-203, 2003.
21. Kassa, J., Krocava, Z., Sevelova, I., Sheshko, V., Kassalova, I. and Neubauerova, V. The influence of single or repeated low-level sarin exposure on immune functions of inbred BALB/c mice. *Clinical Pharmacology and Toxicology* **94**: 139-143, 2004.
22. Kalra, R., Singh, S.P., Razani-Boroujerdi, S., Langley, R.J., Blackwell, W.B., Henderson, R.F. and Sopori, M.L. Subclinical doses of the nerve gas sarin impair T cell responses through the autonomic nervous system. *Toxicology and Applied Pharmacology* **184**: 82-87, 2002.
23. Henderson, R.F., Barr, E.B., Blackwell, W.B., Clark, C.R., Conn, C.A., Kalra, R., March, T.H., Sopori, M.L., Tesfaigzi, Y., Menache, M.G. and Mash, D.C. Response of rats to low-levels of sarin. *Toxicology and Applied Pharmacology* **184**: 67-76, 2002.
24. Williams, A.J., Berti, R., Yao, C., Price, R.A., Velarde, L.C., Koplovitz, I., Schultz, S.M., Tortella, F.C. and Dave, J.R. Central neuro-inflammatory gene response following soman exposure in the rat. *Neuroscience Letters* **349**: 147-150, 2003.
25. Kassa, J., Krocava, Z., Sevelova, I., Sheshko, V., Kassalova, I. and Neubauerova, V. The influence of single or repeated low-level sarin exposure on immune functions of inbred BALB/c mice. *Clinical Pharmacology and Toxicology* **94**: 139-143, 2004.
26. Bentvelsen, F.M., McPhaul, M.J., Wilson, J.D. and George, F.W. The androgen receptor of the urogenital track of the fetal rat is upregulated by androgen. *Molecular and Cellular Endocrinology* **105**: 21-26, 1994.
27. Kelce, W.R., Monosson, E., Gamcsik, M.P., Laws, S.C. and Gray, L.E. Environmental hormone disrupters: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. *Toxicology and Applied Pharmacology* **126**: 276-285, 1994.
28. Bratka-Robia, C.B., Egerbacher, M., Helmreich, M., Mitteregger, G., Benesch, M. and Bamberg, E. Immunohistochemical localization of androgen and oestrogen receptors in canine hair follicles. *Veterinary Dermatology* **13**: 113-118, 2002.
29. Thornton, M.J., Taylor, A.H., Mulligan, K., Al-Azzawi, F., Lyon, C.C., O'Driscoll, J. and Messenger, A.G. The distribution of estrogen receptor β is distinct to that of estrogen receptor α and the androgen receptor in human skin and pilosebaceous unit. *Journal of Investigative Dermatology Symposium Proceedings* **8(1)**: 100-103, 2003.
30. Bratka-Robia, C.B., Egerbacher, M., Helmreich, M., Mitteregger, G., Benesch, M. and Bamberg, E. Immunohistochemical localization of androgen and oestrogen receptors in canine hair follicles. *Veterinary Dermatology* **13**: 113-118, 2002.
31. Tamura, H., Yoshikawa, H., Gaido, K.W., Ross, S.M., DeLisle, R.K., Welsh, W.J. and Richard, A.M. Interaction of organophosphate pesticides and related compounds with the Androgen Receptor. *Environmental Health Perspectives* **111(14)**: 545-552, 2003.
32. Lemaire, G., Terouanne, B., Mauvais, P., Michel, S. and Rahmani, R. Effect of organochlorine pesticides on human androgen receptor activation *in vitro*. *Toxicology and Applied Pharmacology* **196**: 235-246, 2004.
33. Botchkareva, N.V., Botchkarev, V.A., Albers, K.M., Metz, M. and Paus, R. Distinct roles for nerve growth factor and brain-derived neurotrophic factor in controlling the rate of hair follicle morphogenesis. *Journal of Investigative Dermatology* **114(2)**: 314-320, 2000.
34. Botchkarev, V.A., Botchkareva, N.V., Peters, E.M. and Paus, R. Epithelial growth control by neurotrophins: leads and lessons from the hair follicle. *Progress in Brain Research* **146**: 493-513, 2004.
35. Sekowski, J.W., O'Connell, K.P., Khan, A.S., Vahey, M., Nau, M., Khalil, M., Eldefrawi, M.E. and Valdes, J.J. Genetic Mechanisms of Chlorpyrifos Toxicity. *Edgewood Chemical Biological Center Technical Bulletin* **ECBC-TR-210**, October 2001.
36. Delescluse, C., Ledirac, C., de Sousa, G., Pralavorio, M., Lesca, P. and Rahmani, R. Cytotoxic effects and induction of cytochromes P450 1A1/2 by insecticides, in hepatic or epidermal cells:

binding capability to the Ah receptor. *Toxicology Letters* **96-97**: 33-39, 1998.
37. Delescluse, C., Ledirac, C., de Sousa, G., Pralavorio, M., Lesca, P. and Rahmani, R. Cytotoxic effects and induction of cytochromes P450 1A1/2 by insecticides, in hepatic or epidermal cells: binding capability to the Ah receptor. *Toxicology Letters* **96-97**: 33-39, 1998.
38. Gerlardi, A., Morini, F., Dusatti, F., Penco, S. and Ferro, M. Induction by xenobiotics of phase I and II enzyme activities in human keratinocyte line NCTC 2544. *Toxicology in vitro* **15(6)**: 701-711, 2001.
39. Harris, I.A., Siefken, W., Beck-Oldach, K., Brandt, M., Wittern, K.P. and Pollet, D. Comparison of activities dependent on glutathione-S-transfersase and cytochrome P450 1A1 in cultured keratinocytes and reconstructed epidermal models. *Skin Pharmacology and Applied Skin Physiology* **15 (suppl 1)**: 59-67, 2002.